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Thiol-reactive compounds from garlic inhibit the epithelial sodium channel (ENaC)

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ABSTRACT

The epithelial sodium channel (ENaC) is a key factor in the transepithelial movement of sodium, and consequently salt and water homeostasis in various organs. Dysregulated activity of ENaC is associated with human diseases such as hypertension, the salt-wasting syndrome pseudohypoaldosteronism type 1, cystic fibrosis, pulmonary oedema or intestinal disorders. Therefore it is important to identify novel compounds that affect ENaC activity. This study investigated if garlic (*Allium sativum*) and its characteristic organosulfur compounds have impact on ENaCs. Human ENaCs were heterologously expressed in *Xenopus* oocytes and their activity was measured as transmembrane currents by the two-electrode voltage-clamp technique. The application of freshly prepared extract from 5 g of fresh garlic (1% final concentration) decreased transmembrane currents of ENaC-expressing oocytes within 10 min. This effect was dose-dependent and irreversible. It was fully sensitive to the ENaC-inhibitor amiloride and was not apparent on native control oocytes. The effect of garlic was blocked by dithiothreitol and L-cysteine indicating involvement of thiol-reactive compounds. The garlic organosulfur compounds S-allylcysteine, allin and diallyl sulfides had no effect on ENaC. By contrast, the thiol-reactive garlic compound allicin significantly inhibited ENaC to a similar extent as garlic extract. These data indicate that thiol-reactive compounds which are present in garlic inhibit ENaC.

1. Introduction

The epithelial sodium channel (ENaC) is a heteromeric sodium-selective ion channel which classically consists of three subunits, α, β and γ.[1] ENaCs are widely expressed in vertebrate epithelia and represent the rate-limiting step for the transepithelial absorption of sodium. The transepithelial transport of sodium generates osmotic gradients across epithelia, which consequently drive the osmotic transepithelial movement of water. ENaCs are therefore key factors in the regulation of salt and water homeostasis in various organs.

In the kidneys, ENaCs are expressed in the distal nephrons and cortical collecting ducts, where they are responsible for sodium uptake from the primary urine, and are hence involved in the regulation of general body salt and water homeostasis as well as blood volume and blood pressure.[2] In the lungs, ENaCs that are expressed in the airway epithelia regulate the volume and composition of airway lining fluid[3] and facilitate alveolar fluid clearance in the alveolar epithelium.[4] In the intestine, ENaCs are expressed in the aldosterone-sensitive colon and facilitate uptake of sodium and water from the chime.[5]

This indicates that ENaCs are of particular importance for the physiology of several organs and implies that their activity must be precisely regulated. This becomes evident in pathological situations, where an impaired ENaC regulation is associated with human diseases: In the kidneys, a hereditary form of hypertension which is referred to as Liddle's syndrome is due to mutations which lead to an increased number of ENaCs in the plasma membrane.[6] By contrast, loss-of-function mutations of ENaC cause the salt-wasting syndrome pseudohypoaldosteronism type 1.[7] In the lungs, increased ENaC activity in the airways can promote cystic fibrosis-like lung disease,[8–10] whereas ENaC hypoactivity in the distal lung is associated with the formation of pulmonary oedema.[4] In the intestine, impaired ENaC activity is involved in diseases associated with diarrhoea.[11,12]

Discovery of novel compounds, which affect ENaC activity, is relevant for biomedical research on the above-mentioned disorders and is a prerequisite for the development of novel pharmacological tools.

A plant, which has been intensively investigated for its compounds and their pharmacological potential, is garlic (*Allium*

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sativum). Garlic is a major condiment used by households worldwide and has been recognized since centuries as a natural remedy for a variety of diseases, such as infections or cardiovascular diseases.[13] Beneficial effects are attributed to garlic due to its antioxidant, antimutagenic, antiproliferative, antimicrobial or immunomodulatory properties.[13,14] Due to its antibacterial effects, garlic is currently being investigated as a putative therapeutic option in cystic fibrosis.[15] In this context it is important to investigate putative effects of garlic compounds on ENaC since ENaC hyperactivity can also cause cystic fibrosis-like lung disease[9] and decreasing ENaC activity in the airways is considered as a molecular tool for the treatment of this disease. Garlic and its compounds are also thought to reduce risk factors of cardiovascular diseases such as serum lipids, plasma viscosity, platelet aggregation, and blood pressure.[13,16] Based on the link between ENaCs in the kidneys and the long-term regulation of blood pressure[2] it may be hypothesized that blood pressure reduction by garlic might be the result of a putative interaction with renal ENaC activity.

Those examples demonstrate that a variety of cellular and molecular effects are attributed to garlic, which may be relevant for pathological conditions that are also associated with deregulated ENaC activity. Therefore this study investigated the putative impact of garlic and its main compounds on ENaC activity.

2. Materials and methods

2.1. Heterologous expression of ENaC and microelectrode recordings

Defolliculated *Xenopus laevis* oocytes at stages V/VI were injected with cRNA encoding for the human α, β and γ ENaC subunits as previously described.[17] The RNA concentration was 5 ng/μl per subunit, and a volume of 18.4 nl was injected per oocyte. Experiments were performed 24–48 h after injection. Oocytes were placed in a lucite chamber and perfused by a gravity-driven system with oocyte Ringer's solution, containing 90 mM NaCl, 1 mM KCl, 2 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4. Glass capillaries with an outer diameter of 1.2 mm were pulled to microelectrodes and filled with 1 M KCl. The membrane voltage was clamped to −60 mV using a TEVC

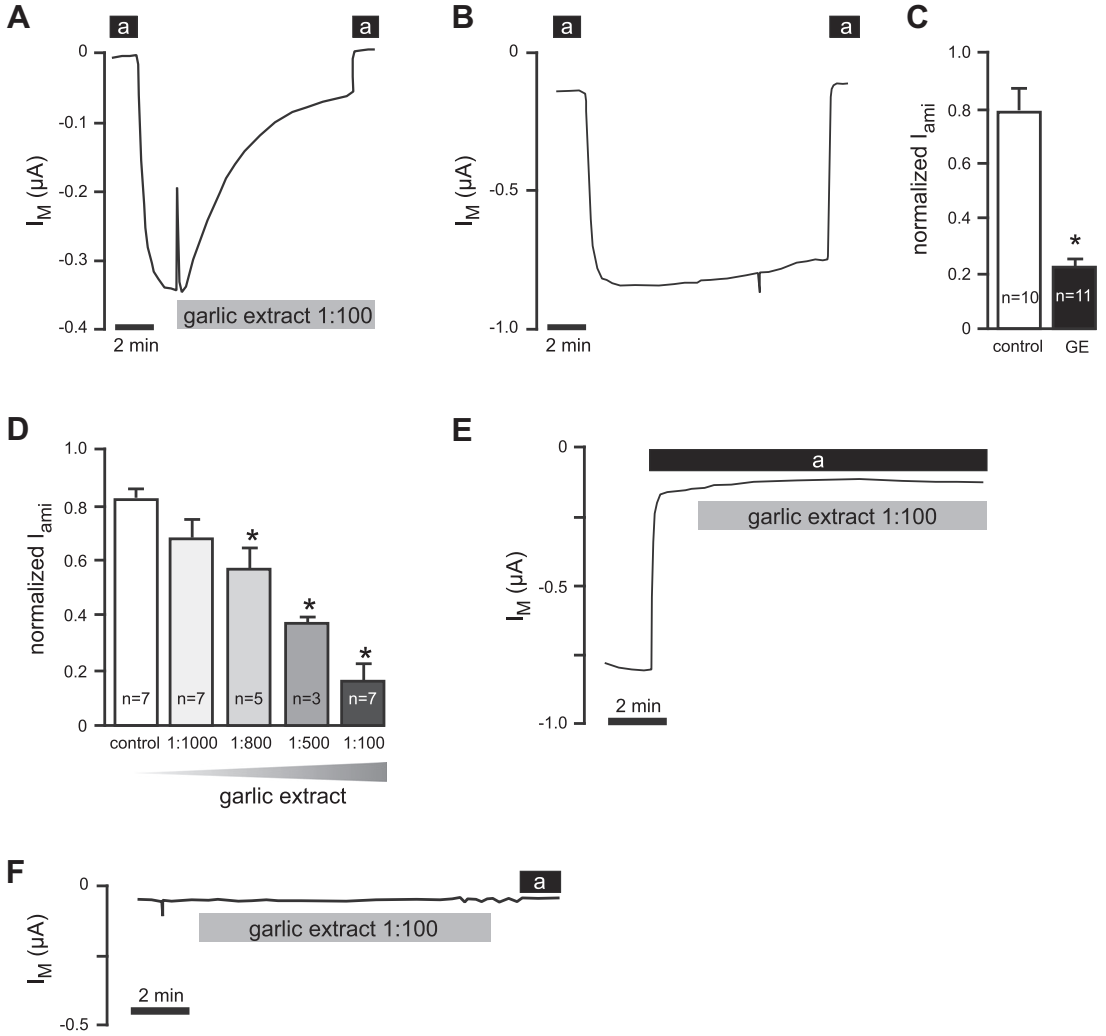

Figure 1. Garlic extract inhibits ENaC. (A) Original transmembrane current (I_M) trace obtained from a microelectrode recording of an ENaC-expressing oocyte. At the beginning of experiments, oocytes were perfused with amiloride (10 μM, 'a', duration indicated by black box). After wash-out of amiloride and reaching plateau conditions, garlic extract (1:100 dilution, grey bar) was applied. After 10 min of exposure to garlic extract, amiloride was applied again. (B) Corresponding control experiment, which followed the same protocol without application of garlic extract. (C) Statistical analysis of experiments as shown in panels A and B. Amiloride-sensitive currents (current differences with and without amiloride) after 10 min of exposure to garlic extract or control conditions were normalized to the baseline amiloride-sensitive currents (normalized I_{ami}). Garlic extract (GE) significantly reduced normalized I_{ami} compared to controls. (D) The effect of garlic extract was dose-dependent. Bars represent normalized I_{ami} obtained from experiments as shown in panel A using different concentrations of garlic extract. (E) In the presence of amiloride (10 μM), garlic extract (1:100) had no effect. (F) Neither garlic extract (1:100), nor amiloride (10 μM) had effects on transmembrane currents (I_M) of non-injected control oocytes.

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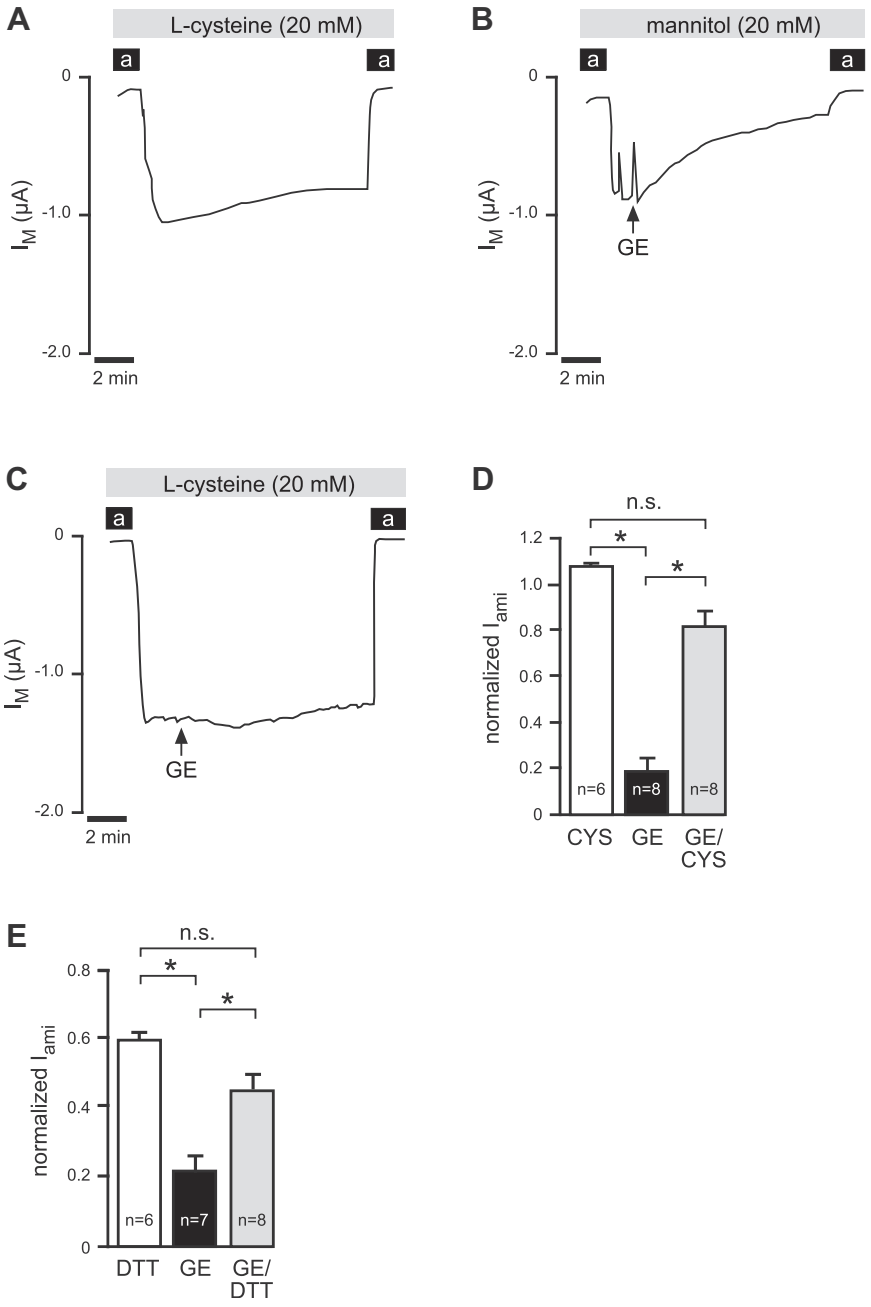

Figure 2. The garlic-mediated inhibition of ENaC is thiol-dependent. (A) Representative current trace. Amiloride-sensitive currents were estimated in the presence of L-cysteine (20 mM). (B) Representative current trace of a positive control experiment. Garlic extract (GE, arrow) was applied to ENaC-expressing oocytes in the presence of mannitol (20 mM). (C) Representative current trace. Garlic extract (GE, arrow) was applied to ENaC-expressing oocytes in the presence of L-cysteine (20 mM). There was no effect of GE on transmembrane currents (I_M) (D) Statistical analysis. Data were obtained from recordings with cysteine alone (CYS; shown in panel A), positive control experiments with garlic extract (GE) under 20 mM mannitol (GE; shown in panel B) and a combination of garlic extract and L-cysteine (GE/CYS; shown in panel C). (E) Statistical analysis of experiments with 10 mM dithiothreitol (DTT), garlic extracts under 10 mM mannitol (GE) and DTT (GE/DTT).

(Two-Electrode Voltage-Clamp) amplifier (Warner Instruments, Hamden, USA), and transmembrane currents (I_M) were continuously recorded with a strip chart recorder (Kipp&Zonen, Delft, The Netherlands).

2.2. Preparation of fresh garlic extract

Bioorganic garlic was obtained from a local supermarket and was distributed by Brio (Verona, Italy) or REWE (Cologne, Germany). Five grams of fresh garlic were pressed into oocyte Ringer's solution with a standard kitchen garlic press. The solution was incubated on ice for one hour and vortexed every 10 min. Subsequently the extract was homogenized and filtered in order to obtain a clear, water-soluble garlic extract. Garlic extract was prepared freshly every day before experiments and diluted in oocyte Ringer's solution to working concentrations (1:100 to 1:1000).

2.3. Chemicals and drug application

Dithiothreitol (DTT) and L-cysteine were obtained from Sigma (Taufkirchen, Germany) and were directly applied to oocyte Ringer's solution in final concentrations of 10 mM and 20 mM, respectively. Control recordings were performed in the presence of analogous concentrations of mannitol (Sigma) in order to exclude osmotic side

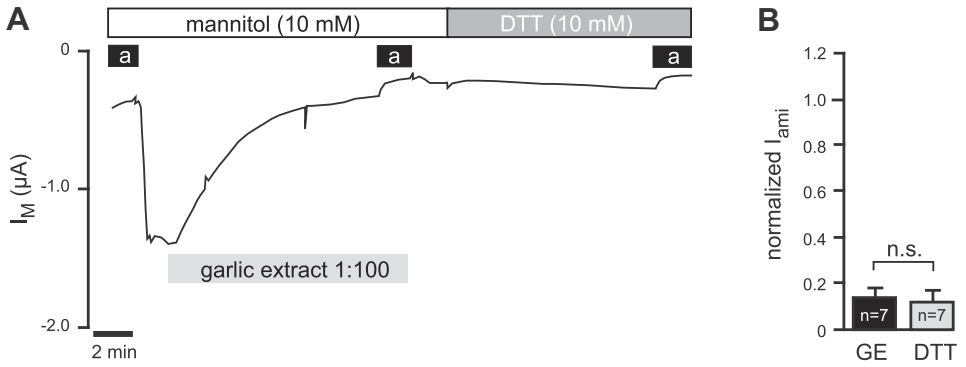

Figure 3. DTT does not restore ENaC activity after exposure to garlic extract. (A) When DTT (10 mM, grey bar) was applied after ENaC inhibition by garlic extract (GE), transmembrane currents (I_M) did not increase back to baseline levels. For osmotic compensation, 10 mM mannitol was present in buffers, when there was no DTT. (B) Statistical analysis from experiments as shown in panel A. Depicted are normalized amiloride-sensitive currents (I_{ami}) with respect to baseline I_{ami}. There were no differences in normalized I_{ami} under garlic extract (GE) and subsequent incubation with DTT. 'a' = amiloride (10 µM).

effects. Allicin and diallyltrisulfide (DATS) were purchased from LKT laboratories (St. Paul, USA). *S*-Allyl-L-cysteine (SAC), alliin, diallylsulfide (DAS), diallyldisulfide (DADS) and amiloride were obtained from Sigma. SAC was freshly dissolved at a final concentration of 1 mM in oocyte Ringer's solution. Alliin was dissolved as a 1 M stock in Ringer's solution and stored at −20 °C. The final concentration in experiments was 1 mM. Allicin was prepared as a stock solution of 10 mM in ethanol and stored at −80 °C. The final concentration of allicin in experiments was 100 µM, which approximately represents the concentration in garlic extract made from 5 g of fresh garlic.[18] DAS and DADS were freshly prepared as 1 M stock solutions in dimethyl sulfoxide (DMSO) before each experiment. DATS was prepared as a 1 M stock solution in ethanol and stored at −80 °C. The final concentration of all diallylsulfides was 1 mM. Adequate controls were performed with solvents (DMSO, ethanol) of the drugs. All drugs were freshly prepared and applied to oocyte Ringer's solution shortly before the experiments. ENaC-expressing oocytes were exposed to drugs by superfusion with a gravity-driven perfusion system. For experiments with allicin and DATS, the drug-containing solution was perfused into the lucite chamber containing the oocytes, and then perfusion was stopped (due to limited amounts of the drugs). After 10 min of incubation, amiloride was perfused into the chambers. Controls were performed accordingly with the drug-solvent ethanol.

2.4. Data analysis and statistics

Values are indicated as mean ± SEM (standard error of the mean). For statistical analysis, mean values of paired experiments (before and after drug exposure on the same oocyte), paired Student's *t*-test was employed. Unpaired experiments (parallel conducted controls and drug exposed oocytes) were compared by unpaired Student's *t*-test. When more than two groups were compared, one-way ANOVA followed by Bonferroni's Multiple Comparison Test was used. Statistical analysis was performed with Microsoft Excel 2003 (Microsoft, Redmond, USA) or GraphPad Prism version 5 (Graphpad Software, San Diego, USA). The number of experiments is indicated by *n* and oocytes from at least two different donor frogs were used for each experiment. *P* values $\leqslant 0.05$ were regarded as significant and marked in the figures with an asterisk (*).

3. Results

3.1. Garlic extract inhibits ENaC

The effect of garlic on human ENaCs was investigated by microelectrode recordings on *Xenopus* oocytes co-expressing the α, β and γ subunits. At the beginning of each experiment, the oocytes were perfused with the specific ENaC-inhibitor amiloride (10 µM; Fig. 1A). After wash-out of amiloride, transmembrane currents (I_M) increased. The differences in I_M under amiloride and after wash-out represent ENaC-mediated amiloride-sensitive currents (I_{ami}). The application of garlic extract (1:100) significantly decreased I_M of ENaC-expressing oocytes from −1.36 ± 0.27 µA to −0.34 ± 0.09 µA ($n = 11$; $p < 0.05$), reaching plateau conditions after ~10 min (Fig. 1A). Subsequently, amiloride was additionally applied, to estimate the remaining I_{ami}. The I_{ami} under garlic extract was largely reduced compared to the baseline I_{ami} (Fig. 1A). Since the effect of garlic was rather slow, corresponding controls were performed on ENaC expressing oocytes, which were not exposed to garlic extract (Fig. 1B). Generally, the I_M of ENaC-expressing oocytes slowly decreased over time, which is a well known phenomenon for ENaCs expressed in *Xenopus* oocytes.[19] Therefore, the fractions of I_{ami} after 10 min were always normalized to the baseline I_{ami}. Garlic extract significantly reduced normalized I_{ami} by 64% when compared to control recordings without garlic extract (Fig. 1C). The effect of garlic extract was not reversible, since I_{ami} did not increase within 10 min after wash-out. The normalized I_{ami} was 0.29 ± 0.04 under garlic extract and 0.22 ± 0.03 after 10 min of wash-out ($n = 9$, $p < 0.05$). Furthermore, the effect of garlic-extract was dose-dependent within dilutions of 1:100 to 1:1000 (Fig. 1D). In the presence of amiloride, the effect of garlic extract was largely blocked (Fig. 1E). Values of I_M were −0.13 ± 0.05 µA before, and −0.08 ± 0.04 µA after 10 min of exposure to garlic extract ($n = 7$; $p < 0.05$). Garlic extract also had no effect on native oocytes (Fig. 1F), which did not express ENaCs (I_M before garlic: −0.029 ± 0.01 µA, after garlic: −0.027 ± 0.01 µA; $n = 8$; $p = 0.52$). Taken together, these data indicate that garlic extract dose-dependently and irreversibly inhibits ENaCs that are heterologously expressed in *Xenopus* oocytes.

3.2. Thiol-reactive substances are involved in ENaC inhibition by garlic extract

Subsequently it was investigated which compounds from garlic are responsible for the observed ENaC inhibition. Garlic contains a variety of organosulfur compounds, which may affect ENaC activity by interaction with cysteine residues of the channel, which play an important role in channel gating.[20] Consistent with this assumption, the effect of garlic extract should be scavenged by high concentrations of free L-cysteine. In the presence of L-cysteine (20 mM) the effect of garlic extract was lost (Fig. 2A–D). Similar results were obtained with the cysteine modifying agent dithiothreitol (DTT, 10 mM; Fig. 2E). These data suggest that thiol-reactive

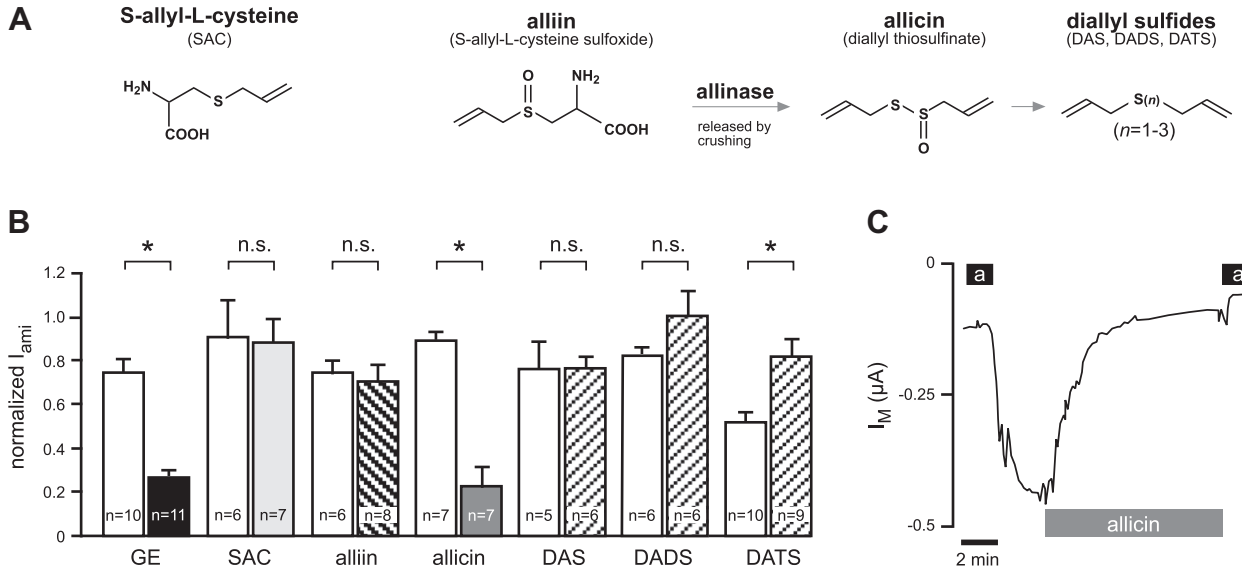

Figure 4. Effects of characteristic garlic compounds on ENaC. (A) Chemistry of characteristic organosulfur compounds of garlic. (B) Effects of garlic extract (GE) and organosulfur compounds on ENaC. Data were obtained from experiments as shown in Fig. 1A either with GE (1:100; for comparison, same data as in Fig. 1C) or with *S*-allyl-L-cysteine (SAC; 1 mM), alliin (1 mM), allicin (100 µM), diallylsulfide (DAS; 1 mM), diallyldisulfide (DADS; 1 mM) or diallyltrisulfide (DATS; 1 mM). Control values originate from experiments with adequate solvent controls (compare Fig. 1B). (C) Original current trace demonstrating the effect of allicin (100 µM) on transmembrane current (I_M) of ENaC-expressing oocytes. 'a' = amiloride (10 µM).

compounds from garlic are likely involved in the inhibitory effect on ENaC. By contrast, when DTT (10 mM) was applied after ENaC-inhibiton by garlic extract, amiloride-sensitive currents did not significantly increase (Fig. 3), which indicates that DTT was not able to restore ENaC activity after exposure to garlic extract.

3.3. The garlic compound allicin inhibits ENaC

Characteristic organosulfur compounds in garlic are *S*-allyl-L-cysteine, alliin, allicin and diallyl sulfides[18] (Fig. 4A). Alliin is located in the cytosol of garlic cells and is converted into allicin by the enzyme allinase. However, allinase is located in the vacuoles of the cells. Therefore, crushing of garlic is necessary to allow contact of allinase with alliin and trigger the conversion to allicin.[18] Allicin and its decomposition derivates, diallyl sulfides (DAS, DADS and DATS)[18], give garlic the characteristic pungent smell. These compounds were screened for their ability to inhibit ENaC (Fig. 4B). *S*-Allyl-L-cysteine, alliin and diallyl sulfides did not inhibit ENaC. However, allicin which was used in concentrations present in the employed garlic extract (100 µM),[18] led to a significant decrease in ENaC activity by 77% (Fig. 4B and C), which was comparable to the general inhibition caused by garlic extract (73%; Figs. 1A and 4B).

Taken together, these data indicate that thiol-reactive organosulfur compounds in garlic, such as allicin, inhibit ENaC.

4. Discussion

The data presented here demonstrate that garlic dose-dependently and irreversibly inhibits ENaCs, which were expressed in *Xenopus* oocytes. This inhibitory effect is sensitive to thiol-modifying agents and can be mimicked by the organosulfur compound allicin in concentrations present in garlic.

Allicin is a highly reactive substance that can rapidly diffuse across cell membranes and has been demonstrated to interact with ion channels, such as TRPA1[21], or intracellular thiol-containing molecules, such as glutathion.[22] The modification of cysteine residues by allicin inhibits enzymes, such as proteases.[23] Thiol-groups are also important for the function of ENaC; each subunit of ENaC contains conserved cysteine-rich domains in the extracellular loop[24] as well as cysteine residues in the intracellular C- and N-termini.[20] Especially the intracellular cysteines are critical for channel function, since mutation or modification of these residues inhibits ENaC activity.[20]

Consistently, it is likely that a cysteine modification by allicin eventually results in an inhibition of ENaC. This is supported by the fact that the general effect of garlic extract is lost in the presence of excess thiol-groups, as is indicated by the experiments with L-cysteine and DTT. Furthermore, the effect of garlic could not be reversed with extracellular application of DTT, which speaks in favor of a modification of intracellular cysteine residues rather than those of the extracellular loops. A modification of intracellular cysteine residues of ENaC subunits by allicin, represents a molecular mechanism how garlic can decrease ENaC activity. Alternatively, allicin may act by modifying enzymes that in turn modulate ENaC activity, which would be consistent with the slow time-course of the garlic effect.

Garlic is recognized since centuries as a natural remedy for a variety of diseases, including cardiovascular diseases.[13] Beneficial effects are attributed to garlic due to its ability to reduce risk factors of cardiovascular diseases such as serum lipids, blood pressure, plasma viscosity and platelet aggregation.[13,16] In particular the impact of garlic on blood pressure regulation is a major focus of ongoing research. There are various studies which investigated effects of garlic on blood pressure and hypertension, including clinical trials.[16,25] However, it is difficult to make a decisive conclusion whether or not garlic indeed reduces blood pressure and/or hypertension, which is mainly due to methodological deficiencies of clinical trials[25] and the lack of detailed molecular data, which would explain putative anti-hypertensive effects of garlic.

The interference of garlic compounds, such as allicin or its metabolites, with ENaC in the kidney would decrease sodium and water retention. This may explain putative beneficial effects attributed to garlic as a natural remedy for diseases that are associated with impaired sodium and water homeostasis, for example salt-dependent hypertension. However, an important point is the bioavailability of garlic compounds. As reported here, among the screened compounds, only allicin was able to mimic the general effect of garlic, whereas the metabolites of allicin, diallsulfides,

failed to do so. Due to the high reactivity of allicin, its half-life in the body is extremely short and there is considerable doubt about allicin being responsible for biological effects of garlic in vivo.[18] Allinase, the enzyme responsible for the formation of allicin, is likely inactivated by pH of gastric fluid.[26] By contrast, allicin is stable in gastric fluid, but unstable in blood.[27] Furthermore, allicin was not detectable in human blood after intake of commercially available garlic products.[18] These data indicate that allicin is unlikely to be a bioavailable compound of garlic and it is questionable if allicin would reach the primary urine and eventually ENaC. Thus it has to be clearly stated that although an inhibition of ENaC by garlic might imply a putative mechanism for its anti-hypertensive effects, the biochemistry underlining the garlic-mediated ENaC inhibition does not favour a physiologically relevant observation. However, allicin-metabolites other than diallylsulfides may be present in body fluids and could affect ENaCs in the kidneys.

Irrespective of its physiological significance, the finding that allicin inhibits ENaC is an interesting starting point for medicinal chemistry. Allicin is a relatively simple organosulfur compound which could be structurally modified in order to enhance its bioavailability. Such modifications should, for example, aim to enhance stability during gastrointestinal passage and increase kidney permeability. An additional challenge would be a precise delivery of allicin to certain cells and tissues in order to counteract toxic side effects due to its high reactivity. In this context it is noteworthy that Miron et al. demonstrated an in situ generation of allicin by conjugation of allinase to an antibody.[28] The concept of delivering enzymes to specific cellular targets via antibodies has been termed antibody-directed enzyme prodrug therapy (ADEPT)[29] and is a promising field in cancer research.[28] This indicates that, despite the instability of allicin, there is a pharmacological potential, which might be of interest in diseases which are associated with increased ENaC activity.

5. Conclusions

The data presented here demonstrate that thiol-reactive compounds from garlic, such as allicin, inhibit ENaC. Although the physiological relevance of this observation has yet to be clarified, these data show that analysing the contents and molecular actions of natural products identifies novel compounds which may be the basis for medicinal chemistry and may have a pharmacological potential for diseases associated with dysregulated ENaC activity.

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